Gynecologic Oncology 136 (2015) 104–111

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Invasion of uterine cervical squamous cell carcinoma cells is facilitated by locoregional interaction with cancer-associated fibroblasts *via* activating transforming growth factor-beta

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HIGHLIGHTS

- TGF-β pathway is distinguished as a driver initiating invasion of cervical squamous cell carcinoma.
- TGF-β is activated through interaction of squamous cell carcinoma cells and cancer associated fibroblasts at tumor invasive front.

ARTICLE INFO

Article history:
Received 26 August 2014
Accepted 21 November 2014
Available online 28 November 2014

Keywords:
TGF-beta
Invasion
Cancer associated fibroblasts
Cervical cancer

ABSTRACT

Objective. Local invasion is a common pattern of spread in uterine cervical squamous cell carcinoma (CSCC). Although transforming growth factor-beta (TGF-β) facilitates invasion of various types of cancer cells, the role of the TGF-β pathway in CSCC is unclear. In this study, we analyzed the role of TGF-β signaling in the progression of CSCC.

Methods. Immunohistochemistry was used to examine the expression of TGF-β pathway molecules in 67 CSCC samples with clinicopathological data. Activation of the TGF-β pathway was investigated following co-culture of CSCC cells and cervical cancer-associated fibroblasts (CCAFs).

Results. Clinicopathological analysis of CSCC samples revealed that prominent expression of TGF-β receptor-2 was more frequent in CSCC with lymphovascular space invasion (LVSI) than without LVSI ($p < 0.01$). Lymph node metastasis was more frequent in cases in which phosphorylated SMAD3 (pSMAD3) was localized exclusively at the boundary of tumor clusters ($n = 9$, $p < 0.05$). Recombinant TGF-β1 increased pSMAD3 expression and enhanced cellular invasion ($p < 0.005$) in CSCC cells, which was attenuated by an inhibitor of the TGF-β receptor ($p < 0.005$). Enhanced pSMAD3 expression and invasion was also observed when conditioned media from CSCC cells co-cultured with CCAFs were administered. Luciferase assays showed that this medium contained a large amount of active TGF-β. Along with TGF-β activation, thrombospondin-1 was upregulated in both CSCC cells and CCAFs, while thrombospondin-1 silencing in either CSCC cells or CCAFs repressed the activity of TGF-β. Thrombospondin-1 was prominently expressed in cases with pSMAD3 boundary staining ($p < 0.05$).

Conclusions. These results suggest that interaction between CSCC cells and surrounding CCAFs activates TGF-β *via* thrombospondin-1 secretion to facilitate CSCC invasion.

Introduction

Despite widespread vaccination against human papilloma virus and periodic cancer screening, cervical cancer remains one of the highest ranking diseases causing mortality in women, and new strategies to treat this disease are urgently needed. Squamous cell carcinoma, the most common histological subtype of cervical cancer, spreads

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http://dx.doi.org/10.1016/j.ygyno.2014.11.075
0090-8258/

principally by migrating into the lymphatics or by invading adjacent soft tissues. Eradication of locoregional lesions is critical but is not attainable in cases with tumor extension into the urinary tract and/or rectum for fear of impairing urinary or bowel function. Even if the extension is more limited, it is also difficult to obtain cancer-free-margins, leading to pelvic recurrence from residual cancer cells. Obstruction of the ureter(s) is not trivial and is observed in 55.8% of advanced cases [1]. It is, therefore, important to develop effective treatments for invasive extension of this disease, and to this end, it is essential to further elucidate the mechanisms of cervical cancer invasion.

Transforming growth factor-beta (TGF-β) is currently known to promote cancer invasion [2]. TGF-β serves as a potent growth inhibitor for normal epithelial cells [3], but malignant transformed cells acquire resistance to the growth inhibitory effect of TGF-β [4]. Moreover, as breast cancer advances, TGF-β acts as a promoter by inducing epithelial to mesenchymal transition (EMT), invasion, and metastasis [5,6]. In gynecologic cancers, activated TGF-β signaling promotes peritoneal dissemination of ovarian cancer [7]. Thus, TGF-β is widely recognized as a key molecule that drives cancer cell progression, but little is known about the role of TGF-β in uterine cervical cancer.

Growing evidence has shown that the stroma around cancer cells plays an important role in cancer progression. Cancer-associated fibroblasts (CAFs) exhibit morphological phenotypes of myofibroblasts and are known to promote cancer progression through interactions with adjacent cancer cells. TGF-β is a key cytokine mediating such interactions [8,9]. However, we presently lack clarification regarding the role of TGF-β in these interactions in cervical squamous cell carcinoma (CSCC).

In this study, we analyze the role of TGF-β signaling in the progression of CSCC. We show that activation of TGF-β induced by the interaction between CSCC cells and CAFs plays a key role in the initiation of tumor metastasis.

Materials and methods

Tissue samples and immunohistochemistry

Tissue samples and clinicopathological information were collected from sixty-seven patients (median age, 54 +/− 23 years) with stage IB–IIB CSCC who underwent radical hysterectomy or trachelectomy in Kyoto University Hospital from January 2003 to July 2010 with written consent under the approval of the ethics committee. Patient characteristics are described in Table 1.

Table 1
Clinicopathological analyses of 67 CSCC patients.

		n	5-year PFS	*p*
Age	<=50 yo	28 (42%)	85.302	—
	>50 yo	39 (58%)	85.322	0.8001
Stage	IB1	38 (57%)	88.610	—
	IB2	6 (9%)	100	0.4226
	IIA1	5 (7%)	100	0.4901
	IIA2	2 (3%)	50.000	0.0145
	IIB	16 (24%)	72.222	0.1667
LVSI	−	22 (33%)	100	—
	+	45 (67%)	78.714	0.0361
Stromal invasion	<1/2-	18 (27%)	100	—
	≥1/2	49 (73%)	79.564	0.4245
Size	<4 cm	55 (81%)	87.893	
	≥4 cm	12 (19%)	74.074	0.0967
Parametrium invasion	−	15 (22%)	87.569	—
	+	52 (78%)	77.037	0.4245
Vaginal margin	>1 cm	62 (93%)	83.826	—
	≤1 cm	5 (7%)	100	0.3502
LNM	−	45 (67%)	92.371	—
	+	22 (33%)	71.753	0.0252

Immunohistochemical staining was performed for six molecules: TGF-β, TGF-β receptor type 1 (TGFBR1), TGF-β receptor type 2 (TGFBR2), pSMAD3, α-smooth muscle actin (α-SMA) and thrombospondin-1 (TSP-1), using the streptavidin–biotin peroxidase complex method as previously reported [7]. The staining methodology is described for each molecule in Supplementary Table 1. Immunostaining was evaluated for intensity and distribution by semi-quantitative scoring as previously described [10] while blinded to clinical data: intensity: 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong); distribution: 0 (no staining), 1 (1–25%), 2 (26–50%) and 3 (51–100%). Staining of TGF-β, TGFBR1, TGFBR2, and TSP-1 was designated as high if cumulative scores were 4 or greater.

Cell lines and culture

Human CSCC cell lines, CaSki, ME-180, QG-U and SKG-IIIa, were obtained from the Riken BioResource Center (Tsukuba, Japan). Primary cervical cancer-associated fibroblasts (pri-CCAFs) were obtained by isolating and mincing the stromal tissue from surgically-removed CSCC with collagenase as previously described [11]. Immortalized cervical cancer-associated fibroblasts (im-CCAFs) and pri-CCAFs were maintained in DMEM (Nacalai Tesque) supplemented with 10% heat-inactivated fetal bovine serum (FBS, v/v; Biowest, France) and penicillin–streptomycin (100 IU/ml penicillin, 100 μg/ml streptomycin; Nacalai Tesque). CSCCs were pretreated with media containing A83-01 (0, 1, 2, or 10 μM, Tocris, Bristol, UK) for 30 min, and were subsequently treated with recombinant TGF-β1 (0, 1, 2, or 10 ng/ml, Peprotech, Rocky Hill, NJ). Protein was extracted from cultured cells using Pierce RIPA Buffer (Thermo, Rockford, IL) supplemented with Protease Inhibitor Cocktail (Nacalai Tesque) and Phosphatase Inhibitor Cocktail (Nacalai Tesque) for Western blotting. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and used for quantitative RT-PCR and microarray analysis.

Stealth RNAi™ siRNA against *TSP-1* (Invitrogen, Carlsbad, CA) or Stealth™ RNAi Negative Control Medium GC Duplex (Invitrogen, Carlsbad, CA) was used for RNA interference or non-targeting assays. Cells were transfected with siRNAs by using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol. siRNA sequences were as follows: siTSP-1,1: CCA GAU CAG GCA GAC ACA GAC AAC A/U GUU GUC UGU GUC UGC CUG AUC UGG, siTSP-1,2: CCA CAG GCC AAA GAC GGG UUU CAU U/A AUG AAA CCC GUC UUU GGC CUG UGG, and siTSP-1,3: UGG CAU CCC UGA GGC AGA UGA AGA A/U UCU UCA UCU GCC UCA GGG AUG CCA.

Cellular functional assays

CSCC cells were treated with/without A83-01 (2 μM) +/− recombinant TGF-β1 (2 ng/ml). Viable cell numbers were determined using a colorimetric assay, and population doubling times were calculated after treatment with TGF-β1. Cellular migration and invasion was assessed by wound healing assays and Boyden-chamber assays as described below.

Wound healing assays

When CSCC cells were 90–100% confluent, “wounds” were introduced by dragging a sterile 1000 μl plastic pipette tip across the cell monolayer. The widths of the scratch lines were measured at five independent locations for each specimen after the treatment. The mean distance filled between the cells most closely spaced on each leading edge of the wound was calculated after 24 h, and the ratio of the distance filled by treated *versus* untreated cells was designated as the migration value.

Invasion assays

After pretreatment with TGF-β1, CSCC cells were collected and suspended in serum-free medium for Boyden-chamber assays. The